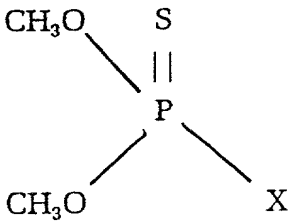
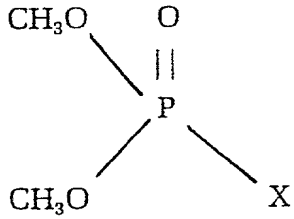
MALATHION CARBOXYLESTERASE

This invention relates to an enzyme (and the nucleic acid sequences encoding this enzyme), termed malathion carboxylesterase (MCE) which is able to efficiently hydrolyse a specific class of organophosphate (OP) insecticides which have the general structures:



(eg. malathion, phenthoate)



(eg. malaoxon, phenthoate oxon)

where X contains one or more carboxylester groups for thion type organophosphates but is unconstrained for oxon type organophosphates.

Residues of organophosphate insecticides are undesirable contaminants of the environment and a range of commodities. Areas of particular sensitivity include contamination of domestic water supplies and soil, residues above permissible levels in various food and fibre exports and contamination of domestic pets. Bioremediation strategies are therefore required for eliminating or reducing these insecticide residues. One proposed strategy involves the use of enzymes capable of immobilising or degrading the insecticide residues. Such enzymes may be employed, for example, in bioreactors through which contaminated water could be passed; in production animal dips to reduce problems with contaminated pasture and run off into water supplies; or in washing solutions after post harvest

disinfestation of fruit, vegetables or animal products to reduce residue levels and withholding times. Suitable enzymes for degrading pesticide residues include esterases. It is desirable that the esterases be relatively specific and hydrolyse the pesticide residues at a rapid rate.

The MCE enzyme has been purified from different malathion resistant strains of *L. cuprina*, RM and der-L (Whyard S., Russell R.J. and Walker V.K., Biochemical Genetics 32: 9, 1994; Whyard S. and Walker V.K., Pesticide Biochemistry and Physiology 50: 198,1994). It is a 60.5 kDa monomer with a K_m for malathion of 11.0 ± 0.4 μM and a V_{max} of 775 ± 28 nmol malathion/min/mg. It also has a high turnover rate for malathion (k_{cat} = 46 min^{-1}).

In order to enable the production of useful amounts of the MCE enzyme the present inventors sought to clone the putative MCE gene from a malathion resistant strain of *L. cuprina(RM-8)* using PCR and cloning techniques.

The MCE gene in *L. cuprina* has been mapped using classical genetic techniques to a position within 0.7 map units from the E3 gene on chromosome 4. The likely homologue of MCE in *Drosophila melanogaster, Mce*, has been mapped to the right arm of chromosome 3 in the vicinity of the genes encoding the major α–carboxylesterase, EST 9, and the orthologue of *L. cuprina* E3, EST23 (Spackman M.E., Oakeshott J.G., Smyth K-A., Medveczky K.M., and Russell R.J., Biochemical Genetics, 32: 39, 1994).

In order to clone the MCE gene from *L. cuprina*, it was decided to use the wealth of molecular genetic techniques available for *D. melanogaster* to clone the MCE homologue and use these clones as probes to isolate the *L. cuprina* genes themselves.

In summary, five esterase amplicons were isolated from *L. cuprina* genomic and cDNA. Four of the five *L. cuprina* amplicons obtained by PCR using cluster specific primers were designated LcαE7, LcαE8, LcαE9 and LcαE10 on the basis of homology to the corresponding *Drosophila* genes. The fifth, Lc#53, could not be assigned with any confidence on the basis of similarity to any of the *Drosophila* genes.

MCE specific activity is highest in the adult head, rather than the thorax or abdomen (Smyth,K-M., Walker,V.K., Russell,R.J. and Oakeshott,J.G. Pesticide Biochemistry and Physiology, 54:48, 1996). On this basis, LcαE7, LcαE8 and LcαE10 were all MCE candidates. Previous

physiological studies of Parker,A.P., Russell,R.J., Delves,A.C. and Oakeshott,J.G.(Pesticide Biochemistry and Physiology 41:305, 1991) have shown that the E3 (LcαE7) enzyme is present in the adult head. Moreover, the LcαE8 and LcαE10 genes are also expressed in the head since PCR using cluster-specific primers were able to amplify these genes from a head cDNA library. PCR failed to detect LcαE9 and Lc#53 in either larval fat body or adult head cDNA and Northern analysis of the D. melanogaster αE9 homologue indicated that this gene was only expressed in embryos. Therefore both LcαE9 and Lc#53 were discounted as candidates for the genes encoding E3 and MCE.

The LcαE8 and LcαE10 genes were initially chosen as prime MCE candidates on the basis of this distribution and due to the fact that it was known that LcαE7 encodes the E3 enzyme involved in diazinon/parathion OP resistance in *L. cuprina* (PCT/AU 95/00016: "Enzyme based bioremediation") and it was thought that malathion resistance and diazinon/parathion resistance were encode by separate genes.

The present inventors have made the surprising finding that it is a variant of LcαE7 which encodes the MCE enzyme. This gene has been expressed *in vitro* and the product shown to have MCE activity. The expressed product can be formulated for use in degrading environmental carboxylester or dimethyl general OPs.

Accordingly, in a first aspect, the present invention consists in an isolated DNA molecule encoding an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, the DNA molecule comprising a nucleotide sequence having at least 60%, preferably at least 80% and more preferably at least 95% homology with LcαE7, in which the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment the present invention the isolated DNA molecule has a sequence as shown in Figure 1 or a sequence which hybridises thereto with the proviso that the protein encoded by the DNA molecule differs from E3 at least in the substitution of Trp at position 251 with an amino acid selected from the group consisting of Leu, Ser, Ala, Ile, Val, Thr, Cys, Met and Gly.

In a preferred embodiment of the present invention the Trp at position 251 is substituted with Leu or Ser.

As is stated above the present invention includes nucleic acid molecules which hybridise to the sequence shown in Figure 1. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3xSCC at about ambient temperature to 65°C, and high stringency conditions as 0.1xSSC at about 65°C. SSC is the abbreviation of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3xSSC is three times as strong as SSC and so on.

In a second aspect the present invention consists in an isolated DNA molecule, the DNA molecule encoding a polypeptide having the amino acid sequence of RM-8Con shown in Figure 1 or the amino acid sequence of MdαE7 shown in Figure 3 in which Trp at position 251 is replaced with Ser.

Homologues of the MCE encoding sequence may also be present in the genome of other insects, and particularly other species of Diptera. Thus, it is to be understood that the invention also extends to these homologues An example of this is provided by the results set out hereunder regarding *Musca* MCE.

The isolated DNA molecules of the present invention may be cloned into a suitable expression vector and subsequently transfected into a prokaryotic or eukaryotic host cell for expression of the enzyme. A particularly suitable system involves baculovirus vectors and an insect cell line.

In a third aspect the present invention consists in a method of producing an enzyme capable of hydrolysing at least one organophosphate selected from the group consisting of carboxylester organophosphates and dimethyl-oxon organophosphates, or an enzymatically active portion thereof, the method comprising transforming a host cell with the DNA molecule of the first aspect of the present invention operatively linked to a control sequence, culturing the transformed cell under conditions which allow expression of the DNA sequence and recovering the produced enzyme, or enzymatically active portion thereof.

It is also envisaged that as an alternative to using the enzyme *per se* as a bioremediation agent the bioremediation agent may be an organism transformed with the DNA encoding the enzyme. In such an arrangement

the organism, transformed such that it expresses the enzyme, would be used as the bioremediation agent.

The invention further relates to methods for eliminating or reducing the concentration of carboxylester or dimethyl-oxon-type organophosphate insecticides residues in a contaminated sample or substance, involving the use of an esterase encoded by an isolated DNA molecule according to the present invention.

In order that the nature of the present invention may be more clearly understood preferred forms will now be described with reference to the following examples and Figures in which:

Figure 1 shows multiple nucleotide alignment of the three malathion-resistant clones (RM8A-C) and their consensus (RM8con) with the reference susceptible clone (Lc743) of LcαE7 (E3). Dots indicate identity with the Lc743 susceptible clone. Below the ruler is the aligned nucleotide sequence and above is the inferred amino acid sequence of Lc743 with the one replacement found in Lc7RM8con indicated in bold text immediately below. Nucleotides are numbered from the predicted start of translation and amino acids from the predicted start methionine. Lc743 5' and Lc743 3' primer sequences are underlined.

Figure 2 shows amino acid alignment of the inferred MdαE7 protein from the Rutgers strain of *Musca domestica* compared to the LcαE7 (E3) protein from the *Lucilia cuprina* Lc743 clone (PCT/AU95/00016 "Enzyme Based Bioremediation"). Sequence comparison shows a 75% identity and 86% similarity between the same length, 570 residue proteins. Arrow indicates the conserved tryptophan residue at position 251 of the alignment.

Figure 3 shows the 1710bp nucleotide coding sequence of the Rutgers strain MdαE7 gene. Also shown is the inferred 570 protein sequence.

Figure 4 shows amino acid alignment of the PCR Ankara strain MdαE7 amplicon and the corresponding region of the RM-8 malathion resistant LcαE7 protein. The structural mutations conferring malathion resistance (serine for MdaE7 and Leucine for LcaE7) are indicated by arrow at residue position 251.

CLONING AND SEQUENCING OF THE MCE GENE FROM A MALATHION RESISTANT STRAIN OF LUCILIA CUPRINA

Two types of change in carboxylesterase activity have been associated with resistance to OP insecticides in the higher Diptera. One type of change results in resistance to OPs like diazinon and parathion, while the other results in resistance to OPs like malathion, with one or more carboxylester groups in addition to the phosphotriester moiety that defines it as an OP (see above).

The two types of change were first described among OP resistant strains of *Musca domestica*. In both types an increased degradation of OPs was associated with reduced ali-esterase activity, where "ali-esterase" refers to enzymes which are major contributors to the hydrolysis of the carboxylester, methyl butyrate, or similar molecules (Oppenoorth, F.J., Entomology Experimental and Applied, 2: 304, 1959; Oppenoorth F.J. and van Asperen, K., Entomology Experimental and Applied 4: 311, 1961). This led to the formulation of the "mutant ali-esterase hypothesis", which proposes that each type of resistance is due to a mutation in a specific carboxylesterase that simultaneously enables it to hydrolyse the phosphoester linkages common to the oxon form of all OPs and decreases its activity toward certain carboxylester substrates (Oppenoorth, F.J. and van Asperen, K.. Science 132: 298, 1960).

Both types of change yielded resistance factors for diverse OPs (except malathion) in the range of about 2-30 fold (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied, 10: 263, 1967). However, the malathion resistant *M. domestica* strains also showed exceptionally high resistance to malathion (generally >100 fold). This high resistance was associated with cleavage of the carboxylester linkages in malathion (ie MCE activity) in addition to the hydrolysis of the phosphoester linkage (OP hydrolase activity). MCE activity accounted for the major breakdown products *in vivo* and *in vitro* (Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969).

No recombination between the two types of resistance or between them and ali-esterase activity was observed among the *M. domestica* strains (Nguy, V.D. and Busvine, J.R., World Health Organisation 22: 531, 1960). This suggests that while they are clearly distinct in respect of OP hydrolase

and MCE activities, the two types of resistance might nevertheless be allelic changes to the same carboxylesterase/ali-esterase gene/enzyme system (Oppenoorth, F. J. and Welling,W., in Insecticide Biochemistry and Pharmacology, Wilkinson, C.F. ed., Plenum Press, New York and London, pp. 507-551, 1976).

A malathion resistance phenotype has also been described in the blowfly, *Chrysomya putoria*, which parallels the malathion resistance phenotype of *M. domestica* in that it is associated with high MCE and low ali-esterase activities (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963; Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). Further evidence for the similarity of the malathion resistance phenotypes in the two species is indicated by the spectrum of OP compounds which synergise malathion. Specifically, among a series of symmetrical trisubstituted phosphorus compounds, the best synergists (eg triphenylphosphate) were common to both species (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967). However, little is known of diazinon/parathion type resistance in *C. putoria*.

The mutant ali-esterase hypothesis has also been invoked to explain diazinon/parathion resistance in *L. cuprina* , because these flies hydrolyse paraoxon more rapidly than susceptible flies (Hughes, P.B. and Devonshire, A.L., Pesticide Biochemistry and Physiology 18:289, 1982) and resistance is associated with reduced carboxylesterase activity. In this case the esterase isozyme E3 from resistant flies is not detected ("non-staining") after polyacrylamide gel electrophoresis (PAGE; Hughes, P.B. and Raftos, D.A., Bulletin of Entomological Research 75: 535, 1985). Evidence for a causal connection between the E3 change and resistance was obtained by EMS mutagenesis of an E3 staining, OP susceptible strain of *L. cuprina* and selection for OP resistant mutants; all resistant mutants recovered had the E3 non-staining PAGE phenotype (McKenzie, J.A., Parker, A.G. and Yen, J.L., Genetics 130: 613, 1992).

Like malathion resistant strains of *M. domestica*, strains of *L. cuprina* that are resistant to malathion exhibit very high resistance factors towards malathion and enhanced MCE activity. Also in common with *M. domestica*, malathion resistant *L. cuprina* generally do not exhibit

diazinon/parathion resistance, and *vice versa* . However, one difference from the situation in *M. domestica* is that the loci encoding the two resistance phenotypes appeared in some experiments to be genetically separable, albeit closely linked (Smyth, K-A., Russell, R.J. and Oakeshott, J.G., Biochemical Genetics 32: 437, 1994; Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G., Pesticide Biochemistry and Physiology 54: 48, 1996)..

An esterase gene cluster containing genes involved in OP resistance has been isolated from *L. cuprina* (Newcomb, R.D., East, P.D., Russell, R.J. and Oakeshott, J.G., Insect Molecular Biology 5: 211, 1996). One of these genes, *LcαE7*, encodes esterase E3 (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, in press), a structural mutation in the active site of which confers diazinon/parathion resistance on *L. cuprina*.. These data are described in a previous patent application PCT/AU 95/00016: "Enzyme based bioremediation", the disclosure of which is incorporated herein by reference.

Below we describe the cloning and sequencing of the *LcαE7* gene from a malathion resistant strain of *L. cuprina*.. We present molecular genetic evidence that this allele of esterase E3 is the MCE gene responsible for malathion resistance in *L. cuprina*.

a) <u>Cloning the malathion resistant allele of *LcαE7*</u>

An RT-PCR (reverse transcriptase - PCR) approach was used to clone a cDNA allele of *LcαE7* from a malathion resistant strain of *L. cuprina* (RM-8) which is homozygous for the fourth chromosome.

Adults from the RM-8 strain were aged for three days before collection and stored at -70° C. RNA was prepared using a modified protocol of Chigwin *et al.* (Chigwin, J.M., Przybyla, A.E., MacDonald, R. J. & Rutter, W.J., 1979, Biochemistry 18, 5294). About 100 adults were thoroughly homogenised in 15ml of solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarkosyl, 0.1M ß-mercaptoethanol) using a Sorvall Omnimix blender. The resulting homogenate was filtered through glasswool and 6 ml layered on top of 5ml of 4.8M CsCl, made up in 10mM Na- EDTA, pH 8, in an SW41 ultracentrifuge tube. These were spun at

35,000 rpm in an SW41 rotor for 16hr at 15° C. The supernatant was removed and the RNA pellet resuspended in 400 μl of DEPC-treated H_2O. The RNA was precipitated by the addition of 800 μl of ethanol and 10μl of 4M NaCl and stored under ethanol at -20°C. Before use the RNA pellet was washed in 75% ethanol and air dried before resuspension in DEPC-treated H_2O.

PolyA$^+$ RNA was prepared from 500 μg of total RNA using affinity chromatography on oligo-dT cellulose (Pharmacia; Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA). The resulting mRNA (1-5μg) was again precipitated, washed and resuspended in 20μl of DEPC-treated H_2O. Oligo-dT primed cDNA was made from 1μg of mRNA using reverse transcriptase (Superscript II, BRL) as per the manufacturers instructions in a 20μl volume reaction. 200ng of cDNA was used as template in each of two PCR reactions using primers designed from the 5' (Lc743 5': 5' atgaatttcaacgttagtttgatggea 3') and complementary 3' (Lc743 3' : 5' ctaaaataaatctctatgtttttcaaac 3') ends of the coding region of the LcαE7 gene. Reactions used Taq DNA polymerase (BRL) and contained 100 pmoles of each primer, 0.2 mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 0.002% Tween 20 (v/v), 1.5 mM $MgCl_2$, and 200 ng of template. Two drops of mineral oil were layered over each 50μl reaction. Six units of Taq enzyme was added after a 5 minute "hot start" at 97°C and was followed by 40 cycles of 35 seconds at 97°C, 1 minute at 60°C and 2 minutes at 72°C. A final cycle of 72°C for 8 minutes was included. The 1.7 kb major product was gel purified and cloned into the EcoRV cleavage site of the pBSK$^-$ (Stratagene) or pGEM-T (Promega) plasmid vectors using conventional cloning techniques (Sambrook, J., Fritsch, E.F., and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, USA).

b) **<u>Sequencing the malathion resistant allele of *LcαE7*</u>**

Methods:

Three clones were chosen for sequencing (RM8-A to -C), all of which were derived from independent PCR reactions. A set of twelve

21-mer sequencing primers (sequence shown below) were designed from the existing LcαE7 sequence:

Table 1

primer seq (5' - 3')	primer name	5' position in Lc743 sequence (Figure 1)
ggatggtgtgcgtgattgttg	7F1	246
aaaaggatgtggtgttgatta	7F2	464
actaatgtcgggtaatgctat	7F3	723
cactatgatgggtaacacttc	7F4	1026
tgttacaggagaaacaccaac	7F5	1203
agaatcgcgtgaatacaaaac	7F6	1467
acggtataccctcaaaactgt	7R1	187
tcccaaacgatattgtatgtt	7R2	504
acatcatgtagtgggtagaag	7R3	685
ccgaggatgtttgggtaagac	7R4	990
tatcagctgttggtgtttctc	7R5	1231
acgcgattctttaggcatacg	7R6	1476

These were used in dye-terminator sequencing reactions (ABI) conducted following manufacturer's instructions in 25μl capillary tubes in a Corbett Research capillary thermal cycler, except that 50pmoles of primer was used per reaction, a "hot start" of 96°C for 3 minutes was included and 30 cycles were completed for each sequencing reaction. Dye primer reactions were also conducted on all clones using the ABI M13 forward and reverse primers as per ABI protocols. Sequencing reactions were resolved by electrophoresis on an ABI 370A automatic sequencing machine as per the manufacturer's instructions. This resulted in both strands being sequenced entirely.

Results:

Figure 1 shows a nucleotide and amino acid alignment of the three resistant clones (RM8 A-C) compared with the reference susceptible clone

(Lc743) of LcαE7. A consensus sequence of the malathion-resistant LcαE7 allele was determined (RM-8con). Differences between resistant clones were assumed to be errors incorporated by the Taq polymerase.

Comparison of the susceptible sequence (Lc743) with that of the malathion-resistant RM-8 consensus sequence (RM-8con) identified only one replacement site difference, a Trp to Leu substitution at amino acid position 251 (nucleotide position 752). The homologous amino acid was highlighted on a three-dimensional model of *T. californica* AChE, revealing that the Leu mutation was situated at the base of the active site gorge, 6.5 Angstroms from the active site Ser.

c) **Sequencing the region surrounding nucleotide 752 from various *LcαE7* alleles**

An esterase structural mutation conferring malathion resistance would be expected to occur in the active site region of the molecule. The Trp to Leu mutation at nucleotide position 752 in LcαE7 is therefore an excellent candidate for the malathion resistance mutation.

The inventors have established a total of 14 strains of *L. cuprina* which are homozygous for chromosome IV and of known malathion resistance status. These lines fall into seven classes on the basis of an RFLP analysis of genomic DNA using the LcαE7 gene as a probe. Nucleotide position 752 was therefore sampled over the entire range of classes.

Methods:

The complete cDNA sequence of the LcαE7 alleles from strains representing several of the classes are available. For example, the sequence of LcαE7 from RM-8 is shown in Figure 1. Moreover, the LcαE7 cDNA sequences from strains LS2 and Llandillo 103, which represent two more classes, are described in patent application PCT/AU 95/00016 ("Enzyme based bioremediation"). The complete LcαE7 cDNA sequence of the Gunning 107 strain, representing a fourth class, is described in J. Trott, B.Agr.Sc Thesis, 1995.

To obtain the sequence of LcαE7 in the region of nucleotide 752 in strains LBB101, Llandillo 104 and Hampton Hill 6.2, representing the

remaining three classes, a PCR approach was taken. Genomic DNA was prepared from either eggs using the method of Davis, L.G., Dibner, M.D., and Batley, J.F., (1986. *Basis Methods in Molecular Biology,* Elsevier Science Publ. Co., New York, Section 5.3), or from adult flies using a C-TAB method (Crozier, Y.C., Koulianos, S. & Crozier, R.H., 1991, Experientia 47, 9668-969). 1μg samples were then used as templates in PCR reactions using 100pmoles of the primers 7F1 and 7R4. Also included in the reactions were 0.2mM of each dNTP, 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$. Two drops of mineral oil were layered over each 50μl reaction. 2.5 units of Taq polymerase was added after a 'hot start' of 97°C for 3 minutes while an annealing temperature of 55° was maintained. An initial extension at 72°C was held for 2 minutes. This was followed by 34 rounds of 97°C for 35 seconds, 55°C for 1 minute and 72°C for 1 minute. A final extension of 72°C for 9 minutes was included. A single product of about 1kb was produced. This was purified for sequencing using QIAquick spin columns (Qiagen), following manufacturer's instructions. 500 ng of template was used in dye-terminator sequencing reactions using the 7F7 (5': 5'tgctgcctctacccactacat 3') and 7R7 (3': 5' cctgtggcttggctttcataa 3') primers as described above.

Results:

Of the seven classes assayed, all five malathion-susceptible strains (LS2, LBB101, Llandillo 104, Gunning 107 and Llandillo 103) possess a G at nucleotide position 752, whereas both malathion-resistant strains (Hampton Hill 6.2 and RM-8) possess a T at this position, resulting in a Trp to Leu substitution at amino acid position 251 (Table 2). The presence of the same structural mutation in two malathion resistant strains with different fourth chromosomes strongly suggests that the mutation is responsible for resistance.

Table 2

Strain	Malathion resistance status	Class	Residue at amino acid position 251[a]
LS2	Susceptible	A	Trp
LBB101	Susceptible	C	Trp
Llandillo 104	Susceptible	B	Trp
RM-8	Resistant	E	Leu
Hampton Hill 6.2	Resistant	F	Leu
Llandillo 103	Susceptible	D	Trp
Gunning 107	Susceptible	G	Trp

[a]Amino acid at position 251 corresponds to nucleotide position 752 in Figure 1.

d) **Cloning and sequencing the orthologous αE7 gene from a malathion resistant strain of *Musca domestica***

As described above, the diazinon/parathion and malathion esterase-mediated OP resistance types exhibit many striking parallels between *L. cuprina, M. domestica and C. putoria* , and are probably caused by functionally equivalent mutations in orthologous genes. The orthologous gene was therefore cloned from the housefly, *M. domestica,* and the region surrounding nucleotide 752 examined for the presence of the malathion resistance mutation in a malathion resistant *Musca* strain.

PCR reactions:

Consensus generic α-esterase primers were designed to the conserved regions of the multiple amino acid alignments of *D. melanogaster* (Robin, C. Russell, R.J., Medveczky, K.M. and Oakeshott, R.J., Journal of Molecular Evolution 43: 241, 1996) and *L. cuprina* (Newcomb, R.D., Campbell, P.M., Russell, R.J. and Oakeshott, J.G. Insect Biochemistry and Molecular Biology, in press) α-esterase genes, and used in a PCR amplification experiment for the recovery of homologous αE7 gene sequence from *M. domestica.*

Genomic DNA was prepared using the Lifton method (Bender, W., Spierer, P. and Hogness, D.S., Journal of Molecular Biology 168: 17, 1989) from adult females of the Rutgers OP resistant housefly strain (Plapp, F.W. Jr., Tate, L.G. and Hodgson, E. 1976. Pestic. Biochem. Physiol. 6:175-182). Rutgers strain genomic DNA was used as the template in a 50µl amplification reaction:

Table 3

	Final concentration/amount
Template DNA	100ng
primer Md1	50pmoles
primer Md2	50pmoles
Buffer	10mM Tris-HCl (pH8.3), 1.5mM $MgCl_2$, 50mM KCl
dNTP's	0.25mM (dATP,dCTP,dTTP,dGTP)
Taq polymerase	1 unit
Total volume	50µl

Primers:

Md1 (35mer)

5' TTCGAGGGIATICCITAYGCIMARCCICCIBTNGG 3'

corresponding to residues 58-69 in *L.cuprina* αE7

Md2 (32mer)

5' ACYTGRTCYTTIARICCIGCRTTICCIGGNAC 3'

corresponding to residues 92-82 in *L. cuprina* αE7

Note: IUB codes used for mixed positions; I = inosine.

PCR conditions over 38 cycles:

95°C	3'						1 cycle
80°C		Hold (addition of 1 unit Taq polymerase)					
95°C	1'	50°C	1'	72°C	1'		1 cycle
95°C	1'	55°C	1'	72°C	1'		35 cycles
95°C	1'	55°C	1'	72°C	4'		1 cycle

Cloning and sequencing PCR amplicons:

The 540bp major product was eluted from an agarose gel, purified using QIAGEN QIAquick PCR purification kit and cloned into the pGEM-T plasmid vector (PROMEGA) using standard techniques (Sambrook, J., Fritsch. E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). The ends of the cloned insert were sequenced using commercially available T7 and SP6 primers and TaqFS dye-terminator technology (ABI) on the Applied Biosystems Model 370A automated DNA sequencer. Translated amino acid sequences were aligned to predicted α-esterase protein sequences using PILEUP from the GCG computer package (Devereux, J., The GCG sequence analysis software package Version 6.0. Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wisconsin, USA, 1989); all proved to be homologous to the sequences of known α-esterase genes from *D. melanogaster* and *L. cuprina*.. The cloned 534bp amplicon showed 76% identity over the equivalent 135 amino acids of the *L. cuprina* αE7 predicted protein sequence.

***Isolation of the complete αE7 gene from* M. Domestica:**

A λDASH (Stratagene) genomic library of the Rutgers strain of *M. domestica* (Koener, J.F., Carino, F.A. and Feyereisen, R. , Insect Biochemistry and Molecular Biology 23:439, 1993) was screened for a full-length genomic clone of *αE7*. Approximately 300,000 plaques were probed with the ^{32}P labelled 534bp amplicon described above. Library screening using conventional techniques (Sambrook, J., Fritsch. E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) was performed at high stringency (50% formamide, 5X SSC, 3X Denhardt's, 0.5% SDS and 10μg/ml salmon sperm DNA at 45°C) and included a final high stringency wash (0.1% SSC, 0.1% SDS at 65°C). Restriction mapping indicated that a single λDASH clone with a 17.5kb genomic insert contained the *αE7* gene. A 4.5kb HindIII fragment was subcloned into the pBSK⁻ vector (Stratagene) and characterised using dye-terminator automatic sequencing technology, as described above. A set

a thirteen sequencing primers were designed and used to interpret the full length genomic sequence:

Table 4

primer name	5' - 3' primer sequence	size	5' position in MdαE7 coding sequence (Figure 3)
T7	end sequencing of 4.5kb pBSK⁻ clone		polycloning site
T3	end sequencing of 4.5kb pBSK⁻ clone		polycloning site
AE7.1	TTTGGTCCCGACTACTTTATGA	22mer	442
AE7.2	TGCCACTTATGAAATCTGTCTGTA	24mer	310
AE7.3	TACATGATGATAACCGAACAGACC	24mer	676
AE7.4	TCGATTATTTGGGTTTCATTTGT	23mer	107
AE7.5	ACAGACAGATTTCATAAGTGG	21mer	288
AE7.6	TTTGCATTCTTTCGGGTGTCA	21mer	913
AE7.7	ATTCGATACCCACATTGATAG	21mer	1016
AE7.8	GGCACTCCCATTTATTTGTAT	21mer	1312
AE7.10	ATGACTTTTCTGAAGCAATTCAT	23mer	1
AE7.11	AAACAATTCCTTCTTTTTATCGA	23mer	1710
AE7.12	GGCATGGAAAACCTCACCTGG	21mer	1558

The predicted coding sequence of 1710bp or 570 amino acids showed a very high 75% identity and 85% similarity to the equivalent full length 570 residues of the αE7 protein from *L. cuprina* (Figures 2 and 3). Southern hybridisation analysis as per standard methods (Sambrook, J., Fritsch. E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) of genomic DNA from the Rutgers strain using the 534bp *αE7* PCR amplicon as a probe and EcoRI, Hind III, Sal I digested DNA, showed a single 4.5kb hybridising band for the HindIII digest, a single 6.0kb SalI band and two EcoRI bands of 1.5kb and 0.8kb. The analysis confirms the restriction pattern interpreted from the sequencing and mapping data of the lambda genomic clone. No other aberrant hybridisation patterns occurred indicating a high probability that αE7 exists as a single copy gene.

Characterisation of a putative αE7 malathion resistant allele of M. domestic:

Genomic DNA extracted (Bender, W., Spierer, P. and Hogness, D.S., Journal of Molecular Biology 168: 17, 1989) from single adult female flies of the highly malathion resistant Ankara strain (Sisli, M.N., Bosgelmez, A., Kocak, O., and Porsuk, H. 1983. Mikrobiyol Bul. 17:49-46) was used for sequence characterisation of a putative malathion resistance allele. A series of PCR reactions were performed using single fly genomic DNA for the characterisation of allelic variants of the *αE7* gene. PCR amplification using conditions described above, with the specific housefly AE7.5 and AE7.6 primer pair, produced single amplicons of approximately 760bp. This amplicon encompasses the highly conserved region involved in the catalytic site of the enzyme, coding for residues 96-304 of the translated sequence, including the site of the Trp to Leu mutation at amino acid residue 251 associated with malathion resistance in *L. cuprina* (Figure 4). Cloning and sequencing of PCR amplicons (described earlier) from nine individual flies showed that the Ankara strain segregates for two allelic variants of the αE7 gene: one has a Trp residue at amino acid position 251, whereas the other has a Ser at this same position. This replacement is synonymous with the Trp to Leu substitution involved in malathion resistance in *L. cuprina*. Both leucine and serine replace a bulky tryptophan residue within the active site and we therefore propose that this change accounts for the observed changes in the kinetic properties of the enzymes towards carboxylesters and OPs. In a similar manner it is believed that the substitution of the bulky Trp residue with other smaller residues such as Ala, Ile, Gly, Val, Thr, Cys and Met will have a similar effect. The finding of these similar active site mutations in malathion resistant strains of both *Lucilia* and *Musca* further supports our conclusion that these mutations are responsible for malathion resistance in these species.

HYDROLYTIC ACTIVITY OF THE EXPRESSED PRODUCTS OF THE SUSCEPTIBLE AND MALATHION RESISTANT ALLELES OF *LcαE7*

Below we describe the activities of the expressed products of the susceptible and malathion resistant alleles of *LcαE7* for various

carboxylester and OP substrates. The results suggest a possible mechanism for malathion resistance in *L. cuprina* as a result of the mutation at nucleotide 752 in the *LcαE7* gene

a) ***In vitro* expression**

The *in vitro* expression of the OP susceptible allele of *LcαE7* (clone Lc743) is described in patent application PCT/AU 95/00016 ("Enzyme based bioremediation").

The malathion resistant *LcαE7* full-length cDNA was cloned into the baculovirus transfer vector, Bacpac 6 (Clonetech) 3' of the polyhedrin promoter. Transfections were conducted using a lipofection method with DOTAP (Boehringer Mannheim) as per King and Possee (The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992). One μg of DNA of each of the resulting constructs together with 200 ng of Bacpac 6 baculovirus DNA (Clonetech), linearised by digestion with the restriction enzyme BSU 36I (Promega), was incubated in a solution of HBS (hepes buffered saline) containing 15% DOTAP (Boehringer Mannheim) in a polystyrene container at room temperature for 10 minutes. The solution was then used to transfect a single well of a six well tissue culture plate pre-seeded 2 hrs previously with 10^4 Sf9 (*Spodoptera frugiperda*) cells in 1.5 mls Grace's medium (King and Possee, The Baculovirus Expression System: A Laboratory Guide, Chapman & Hall, London, 1992). After 12 hours, the medium was replaced with 3 mls of Grace's medium containing 10% fetal calf serum. Construct plus DOTAP, linearised virus plus DOTAP and DOTAP only controls were conducted in parallel with transfections. The transfections were harvested 4-5 days after infection and the cells isolated by centrifugation at 500g for 5 minutes. Aliquots of the resulting cell pellets were immediately stored on ice, resuspended in 10mM imidazole-HCl buffer, pH 7.0, containing 0.5% Triton X-100. Final protein concentrations in these cell extracts were between 5 and 40 mg/ml. Aliquots of the cell extracts were stored at -70°C prior to enzyme assays.

b) **Malathion hydrolysis**

Methods:

MCE activity was assayed using the partition method of Ziegler, R., Whyard, S., Downe, A.E.R., Wyatt, G.R. and Walker, V.K., Pesticide Biochemistry and Physiology, 28:279 (1987) as modified by Whyard, S., Russell, R.J. and Walker V.K. Biochemical Genetics 32:9 (1994). Cell extracts were diluted 300-fold in 10mM imidazole-HCl, pH 7.0, and 150µl aliquots were placed in triplicate microfuge tubes. Reactions were started by the addition of 1µl ethanol containing [^{14}C]-malathion [Amersham; 103 mCi/mmole, 280nCi, labelled at both the methylene carbons of the succinate moiety, adjusted to 15mM (or 375µM - 15mM for kinetic experiments) by the addition of unlabelled malathion (99%; Riedel-de-Haèn Ag., Seelze, Germany)]. The assay mixture was incubated at 25°C for 10 minutes, then 300µl of dilution buffer was added and the undegraded malathion extracted three times with 600µl of chloroform. The concentration of carboxylic acids of malathion in 300µl of aqueous phase was determined by liquid scintillation. Protein concentrations in the cell supernatants were determined using the Biorad Protein Assay Kit by the method of Bradford, M., Analytical Biochemistry 72:248 (1976) with bovine serum albumin as the standard. Boiled enzyme controls were performed routinely. The specific MCE activity of an extract of cells infected with non-recombinant baculovirus was at least 700-fold lower than that of cells infected with baculovirus encoding OP-susceptible or malathion-resistance alleles of LcαE7. This slight MCE activity that was not due to alleles of LcαE7 was deemed a very minor source or error and subsequently ignored.

Results:

Using initial concentrations of malathion between 2.5 and 100 µM, MCE encoded by malathion resistant and susceptible alleles of *LcαE7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the molarity of the *LcαE7* products in their respective cell extracts. The molarity of susceptible *LcαE7* product in the cell extract was determined by

titration with paraoxon as previously described in Newcomb, R. D., Campbell, P.M., Russell, R.J. and Oakeshott, J. G., Insect Biochemistry and Molecular Biology (in press). The molarity of the product of the malathion resistant allele of *LcαE7* was determined similarly, except that triphenylphosphate (TPP; 1 to 10 x 10^{-8}M) was used instead of paraoxon. In a control experiment TPP (8 x 10^{-8}M) was preincubated with cell extract in triplicate for 15, 30 or 45 minutes prior to addition of the substrate malathion. There was no significant difference between the residual MCE activity at each of the preincubation times, indicating firstly that the inhibition of MCE by TPP had gone to completion and secondly, that TPP was not being turned over by MCE.

The kinetic parameters for malathion hydrolysis for the products of the malathion resistant and susceptible alleles of *LcαE7* are:

Table 5

Expressed LcαE7 Gene Product	K_m (μM)	K_{cat} (min^{-1})
Malathion susceptible (strain LS2)	200±30	70±11
Malathion resistant (strain RM-8)	21±1	43±1

The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies (11±0.4 μM and 46±2 per min, respectively; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

c) **Sensitivity of MCE activity to TPP**

High sensitivity to inhibition by TPP is a distinctive characteristic of the MCE activity associated with malathion resistance in *M. domestica*, *C. putoria* and *L. cuprina* , consistent with potent synergism of malathion by TPP in resistant strains of these species (Shono, T., Applied and Entomological Zoology 18: 407, 1983; Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969; Hughes, P.B., Green, P.E. and Reichmann, K.G., Journal of Economic Entomology 77: 1400, 1984: Smyth, K-A., Walker, V.K., Russell, R.J. and Oakeshott, J.G.,

Pesticide Biochemistry and Physiology, 54: 48, 1996). MCE activity encoded by the malathion resistant allele of *LcαE7* is potently inhibited by TPP, as indicated by stoichiometric inhibition of the enzyme at concentrations below 10^{-7}M (see above).

d) α-Naphthyl acetate hydrolysis

Methods:

The initial rates of reactions between cell extracts containing the expressed products of the malathion resistant and susceptible *LcαE7* alleles and α-naphthyl acetate (α-NA) were determined at 25°C using a recording spectrophotometer and the method of Mastrapaolo and Yourno (Analytical Biochemistry 115: 188, 1981). 6-200μM α-NA dissolved in 10μl of 2-methoxyethanol was added to 0.1 M Tris-HCl pH 8.0 (980 μl) in a quartz cuvette. α-Naphthyl acetate is slowly hydrolysed in water so a background rate was recorded before starting the enzymic reaction by the addition of 10 μl of diluted cell extract. Control reactions were performed with extracts of both uninfected cells and Bacpac 6 infected cells. These controls exhibited negligible enzymic hydrolysis.

Results:

Using initial concentrations of α-NA from 6 to 200 μM, the enzymes encoded by the malathion resistant and susceptible alleles of *LcαE7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the molarity of the *LcαE7* products in their respective cell extracts (determined above).

Table 6

Expressed LcαE7 Gene Product	K_m (μM)	K_{cat} (min^{-1})
Malathion susceptible (strain LS2)	70±5	11,000±300
Malathion resistant (strain RM-8)	150±50	2270±30

The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies (167±14 μM and 2063 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

e) <u>α-Naphthyl butyrate hydrolysis</u>

Methods:

As described above for α-NA hydrolysis except that 6-200 μM α-naphthyl butyrate (α-NB) was used instead of α-NA.

Results:

Using initial concentrations of α-NB from 6 to 200 μM, the enzymes encoded by the malathion resistant and susceptible alleles of *LcαE7* exhibited a good fit to Michaelis-Menten kinetics. K_m and V_{max} were calculated for both enzymes. K_{cat} was then calculated from the V_{max} and the molarity of the *LcαE7* products in their respective cell extracts (determined above).

Table 7

Expressed LcαE7 Gene Product	K_m (μM)	K_{cat} (min^{-1})
Malathion susceptible (strain LS2)	20±5	18,000±2,000
Malathion resistant (strain RM-8)	29±4	9,000±400

The K_m and K_{cat} for the malathion resistant product are in reasonable agreement with those determined for the MCE enzyme purified from malathion resistant flies (39±4 μM and 3700 per min; Whyard, S. and Walker, V.K., Pesticide Biochemistry and Physiology, 50: 198, 1994).

f) General OP hydrolysis

In *M. domestica* there is a pattern of cross-resistance among OPs (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967) such that parathion/diazinon resistant flies generally exhibit greater resistance factors towards OPs with two ethoxy groups attached to the phosphorus atom ('diethyl OPs') rather than two methoxy groups ('dimethyl OPs'). The converse pattern (ie greater resistance to dimethyl OPs) was observed for malathion resistant strains of *M. domestica* and *C. putoria* (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). This dimethyl OP preference applies both to malathion analogues (with carboxylester groups) and general OPs (without carboxylester groups). The implication of these studies is that there is a general OP hydrolase activity intimately associated with malathion type resistance and that this OP hydrolase exhibits a preference for dimethyl OPs.

There are insufficient published data to determine whether such a dimethyl/diethyl OP cross-resistance pattern occurs in *L. cuprina*. Here we determine firstly that there is such a cross resistance pattern and secondly that the enzyme encoded by the malathion resistance allele of *Lcα*E7 has hydrolytic activity against OPs which lack carboxylester groups.

Methods:

i) Toxicology:

The following organophosphorus compounds were used: diazinon (O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate, 91%, Mallinckrodt), parathion-methyl (O,O-dimethyl O-4-nitrophenyl phosphorothioate, 97.0%, Bayer), parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate, 99%, Pestanal grade, Riedel-de-Haën), fenthion (O,O-dimethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, 98.8%, Bayer), fenthion-ethyl (O,O-diethyl O-[3-methyl-4-(methylthio)phenyl] phosphorothioate, a gift from Dr. G. Levot), dichlorvos (2,2-dichlorovinyl dimethyl phosphate, 99%, Chem Service), diethyl-dichlorvos (2,2-dichlorovinyl diethyl phosphate, a gift from Dr. J. Desmarchelier), di-

isopropyl-dichlorvos (2,2-dichlorovinyl di-isopropyl phosphate, a gift from Dr. J. Desmarchelier), malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-dimethyl phosphorodithioate, technical grade, Nufarm), isopropyl malathion (S-1,2-bis(ethoxycarbonyl)ethyl O,O-di-isopropyl phosphorodithioate, a gift from Dr. J. Desmarchelier).

The toxicity of OPs in adult female *L. cuprina* was determined 3 or 4 days post-eclosion by application of OPs to the scutellum in 0.7 μl dioctylphthalate (Busvine, J.R., Bell, J.D. and Guneidy, A.M., Bulletin of Entomological Research 54: 589, 1963;; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969). Each OP was applied to at least 20 flies at each of at least 5 different concentrations, spanning the dose causing 50% mortality (LD_{50}). Control groups were treated with solvent but no OP. Mortality was determined 24 hours later. Data were fitted to the probit curve using the Probit Or LOgit (POLO-PC) computer program (LeOra Software, 1987). This program corrects for natural mortality which was <5%. The statistic *g* ("index of significance for potency estimation") was always less than 0.5 for the 95% confidence limits of LD_{50}.

ii) Chlorfenvinphos hydrolysis assay:

Enzyme samples were diluted in 0.1 M imidazole-HCl buffer pH 7.0 ("imidazole buffer") to a final volume of 50 μl. Reactions were started by the addition 0.5μl of (^{14}C-ethyl)-chlorfenvinphos (CVP, 306.5 MBq/mmole, Internationale Isotope Munchen) from a 7.5mM stock solution in ethanol. The reaction was incubated at 30°C and stopped by the addition of 300 μl dichloromethane and 150 μl of water followed by vigorous vortex mixing. The reactions were centrifuged to separate phases and 150 μl of the upper, aqueous phase was taken for scintillation counting to determine the amount of ^{14}C-diethylphosphate produced by hydrolysis of CVP. Incubations with boiled enzyme were also performed to control for non-enzymic hydrolysis of CVP.

Results:

I) Toxicology:

LD_{50}s of 10 OPs were determined for the Woodside 5.2 strain (homozygous for a malathion resistance allele of *LcαE7*) and the Llandillo 103 strain (homozygous for a parathion/diazinon resistance allele of *LcαE7*). LD_{50}s were also determined for the OP susceptible LS2 strain in order to calculate resistance factors (Table 8). Woodside 5.2 flies exhibited about two- to five-fold greater resistance factors towards the dimethyl OPs, parathion-methyl, fenthion and dichlorvos than to their diethyl analogues, parathion, fenthion-ethyl and ethyl dichlorvos. Conversely, Llandillo 103 flies exhibited about two-fold greater resistance factors towards the diethyl OPs, parathion and ethyl dichlorvos, than to their dimethyl analogues, parathion-methyl and dichlorvos. However, there was no significant difference between the resistance factors of Llandillo 103 flies for fenthion and fenthion-ethyl.

Among four diethyl OPs, Llandillo 103 flies have higher resistance factors than Woodside 5.2 flies (except fenthion-ethyl with similar resistance factors; Table 8). In contrast, Woodside 5.2 flies have higher resistance factors than Llandillo 103 for each of four dimethyl OPs. Thus both strains exhibit general OP resistance of similar potency, albeit with a bias towards either dimethyl or diethyl OPs.

Neither resistant strain exhibited more than 3-fold resistance to the di-isopropyl analogues of dichlorvos or malathion (Table 8).

Comparable data from *M. domestica* are available for seven of the test compounds. In each case resistance factors are similar in strains of the two species exhibiting parallel resistance types (Table 8).

Table 8 OP cross-resistance patterns in adult *L. cuprina* with comparisons to *M. domestica*.

OP Compounds[1]	LD_{50}[2] LS2 (OP Susceptible)	LD_{50} Llandillo 103 (Dz/para Resistant)	LD_{50} Woodside 5.2 (Mal Resistant)	RF[3] (Dz/para R)	RF (Mal R)
Diazinon (E)	57 (40-79), 3.5 ± 0.5	550 (490-630), 4.9 ± 0.7	270 (240-300), 5.1 ± 0.6	10 (20)	5 (2)
Parathion-methyl (M)	16 (11-20), 6.5 ± 0.8	185 (141-244), 3.4 ± 0.5	430 (390-490), 9.7 ± 1.5	12 (9)	27 (10)
Parathion (E)	52 (48-55), 9.2 ± 1.1	1050 (890-1280), 6.8 ± 1.2	290 (270-310), 13.3 ± 2.3	20 (35)	6 (3)
Fenthion (M)	61 (42-87), 5.2 ± 0.7	210 (180-240) 8.7 ± 1.5	320 (210-490) 3.9 ± 0.7	3 (3)	5 (7)
Fenthion-ethyl (E)	330 (290-370) 7.4 ± 1.1	690 (570-830) 8.7 ± 1.5	730 (660-870) 13.4 ± 2.6	2	2
Dichlorvos (M)	41 (35-51), 8.2 ± 1.1	150 (95-190), 5.1 ± 0.8	270 (210-340) 6.3 ± 1.0	4 (3)	7 (6)
Ethyl Dichlorvos (E)	360 (300-420), 5.0 ± 0.8	2370 (2320-2410), 54 ± 14	1100 (700-1500) 4.9 ± 1.2	7	3
Isopropyl Dichlorvos (P)	3500 (2200-4800), 4.1 ± 0.6	4600 (3400-5900) 4.1 ± 0.5	10200 (8800-12000) 8.1 ± 1.4	NS	3
Malathion (M)	550 (480-610), 6.4 ± 1.2	490 (360-600) 4.2 ± 0.9	4	NS (2)	>130 (157)
Isopropyl Malathion (P)	3600 (2700-4900), 6.3 ± 0.7	4900 (3700-6200), 8.5 ± 1.7	6400 (5900-7100), 10.1 ± 1.8	NS	1.8 (4)

1. Dimethyl (M), diethyl (E) or di-isopropyl (P) OPs.
2. LD_{50} (ng/fly) with 95% confidence limits, slope and standard error of the probit regression line.
3. Resistance Factors: ratio of the LD_{50} of Llandillo 103 or Woodside 5.2 with the LD_{50} of LS2. "NS" indicates an RF not significantly different from unity. Resistance factors of *M. domestica* of the appropriate resistance type are shown in parentheses (Bell, J.D. and Busvine, J.R., Entomology Experimental and Applied 10: 263, 1967; ; Townsend, M.G. and Busvine, J.R., Entomology Experimental and Applied 12: 243, 1969).
4. No mortality at this dose.

ii) Chlorfenvinphos hydrolytic activity of whole fly homogenates and expressed *LcαE7* gene products:

Whole fly homogenates of malathion resistant (strains RM-8, 60NE 1.1, 4.2, Beverly 6.2, Hampton Hill 6.1,Hampton Hill 6.2, Woodside 5.2, Rop Rmal 1, M22.2 6.3, M27.1 4.1), diazinon resistant (Gunning 107, Inverell 22, Q4, RM2.6, Llandillo 103, Sunbury 5.2) and susceptible (LBB 101, Llandillo 104, LS2) strains of *L. cuprina* were tested for esterase-mediated hydrolysis of CVP, a general OP (ie not a malathion-type OP). All 10 malathion resistant strains had greater CVP hydrolytic activities (1.5 -3.0 pmol/min/mg) than the 3 susceptible strains (0.5 -1.0 pmol/min/mg, but less activity than the 6 diazinon resistant strains (8.2 - 30.0 pmol/min/mg).

The expressed product of the malathion resistant LcαE7 allele was tested for CVP hydrolytic activity. Turnover of 75μM CVP was about 1.2 hour^{-1}, which is approximately 50-fold less than that of the diazinon resistant (RM2-6) LcαE7 gene product but much greater than that of the OP-susceptible (LS2) gene product, for which CVP activity was undetectable. [The CVP hydrolytic activity of the gene products of the RM2-6 and LS2 alleles of LcαE7 are described in patent application PCT/AU 95/00016 : "Enzyme based bioremediation"].

g) **Conclusions**

1. We have discovered that dimethyl versus diethyl patterns of OP cross-resistance among strains of *L. cuprina* parallel those of OP resistant strains of *M. domestica* and *C. putoria* . The two OP resistance types are equally potent and general among most OPs (excluding malathion), albeit with a dimethyl or diethyl OP preference.

2. Diethyl OP hydrolytic activities encoded by the OP susceptible allele (nil), the malathion resistant allele (1.2 hour^{-1}) and the diazinon/parathion resistant alleles of *LcαE7* (~1 min^{-1}) parallel the diethyl OP hydrolytic activities in homogenates of OP susceptible (low), malathion resistant (intermediate) and diazinon/parathion resistant (high) *L. cuprina* strains.

3. Taking points 1 and 2 together we propose that the dimethyl versus diethyl pattern of general OP cross resistance reflects the substrate specificity of the general OP hydrolase activities encoded by the two alternative OP resistance alleles of the *αE7* gene. Thus we expect that products of malathion resistance alleles of *αE7* genes from *L. cuprina* and *M. domestica* will exhibit dimethyl OP hydrolysis with kinetics that are as favourable for bioremediation as the diazinon/parathion resistance *αE7* alleles are for diethyl OPs.

4. The enhanced MCE activity of the product of the malathion resistance alleles of *αE7* genes causes flies to survive more than 100-fold greater doses of malathion. The MCE activity is enhanced in two ways. Firstly, it has more favourable kinetics for malathion breakdown than that of the susceptible allele (K_{cat}/K_m is 6-fold greater). Secondly, it has acquired general OP hydrolase activity. The latter is important for both resistance and bioremediation because it enables the enzyme to recover its MCE activity after phosphorylation/inhibition by the 'activated' or 'P=O' form of OP insecticides. P=O OPs are encountered in an insect because they are generated by an insect's metabolism. For bioremediation the OP hydrolase is required for two reasons; firstly, to hydrolyse general OPs where these are the main contaminant, and secondly, to ensure that malathion hydrolysis by the enzyme will continue in the presence of minor contamination with 'P=O' OPs.

ALLELISM OF THE MALATHION AND DIAZINON/PARATHION RESISTANCE PHENOTYPES IN *L. CUPRINA*

It is clear from the above molecular genetic and biochemical data that malathion resistance in *L. cuprina* is conferred by a structural mutation in the active site of *Lcαe7* (esterase E3), the same gene that is involved in resistance to diazinon/parathion type OPs. However, two classical genetic studies have detected a small number of presumptive recombinants between the malathion and diazinon resistance phenotypes in *L. cuprina*. which would suggest that they are separate, albeit closely linked, genes (Raftos, D.A. and Hughes, P.B., Journal of Economic Entomology 77: 553. 1986;

Smyth, K-A., Russell, R.J. and Oakeshott, J.G., Biochemical Genetics 32: 437, 1994).

The availability of frozen extracts from three of the five presumptive recombinants generated by the study of Smyth *et al.*. (1994) enabled the authors to test them directly for the predicted genotypes using PCR techniques. In this particular study, diazinon resistant males (strain Q4) were crossed to malathion resistant females (strain RM-8) and the F1 females backcrossed to Q4 males. Progeny were scored for the E3-non-staining, high MCE phenotypes as indicative of resistance to both diazinon and malathion. Five presumably recombinant individuals showing the MCE high/E3 non-staining phenotype were recovered from 692 backcross progeny; none were recovered with the reciprocal MCE low/E3 staining phenotype, which would presumably be susceptible to both malathion and diazinon.

Methods:

The regions of the *LcαE7* gene surrounding the diazinon resistance (Gly to Asp substitution at position 137) and malathion resistance (Trp to Leu at position 251) mutations were amplified from each of the three available extracts from the Smyth *et al* ., (1994) study, using the 7F1/7R2 and 7F7/7R4 primer sets, respectively. The PCR conditions and primers (except 7F7) are as described above, except that an annealing temperature of 55°C and a buffer supplied by the manufacturer of Taq DNA polymerase (BRL; 0.2 mM of each dNTP, 20 mM Tris-HCl, pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$) were used. The 7F7 primer has the sequence: 5' tgctgcctctacccactacat 3í and its 5' position in the Lc743 sequence is nucleotide 660 (see Figure 1).

The 330nt fragment of *LcαE7* generated by the 7F7/7R4 primer set contains an RFLP polymorphism specific for each of the Q4 and RM-8 alleles: an *Nco1* cleavage site at nucleotide position 752 marks the Q4 allele (this polymorphism is at the site of the Trp to Leu mutation responsible for malathion resistance), while a *Bgl1* site at position 796 characterises the RM-8 allele. Therefore, in order to identify the Q4 and RM-8 alleles in each extract, PCR products were digested directly with each restriction enzyme and the products sized by agarose gel electrophoresis, using standard techniques (Sambrook, J., Fritsch. E.F. and Maniatis, T., Molecular Cloning:

A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989). Controls contained PCR products generated from Q4 and RM-8 genomic DNA.

No such convenient RFLP polymorphisms were contained in the 326nt fragment amplified by the 7F1/7R2 primer set (this fragment contains a 68nt intron at nucleotide position 360). However, three nucleotide polymorphisms distinguish the Q4 and RM-8 fragments: an A to T substitution at nucleotide position 303 , T to C at position 345 and G to A at position 410 in the Q4 sequence (the latter substitution is responsible for diazinon/parathion resistance). PCR products were therefore cloned into the pGEM-T vector (Promega) using standard techniques (Sambrook, J., Fritsch. E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, USA, 1989) and individual clones sequenced using commercially available SP6 and T7 primers and dye terminator technology, as described above.

Results:

Digestion of the PCR products generated by the 7F7/7R4 primer set with either *Nco1* or *Bgl1* revealed the presence of only the RM-8 chromosome in all three extracts (ie PCR products could be cleaved by *Bgl1* and not *Nco1*, whereas the products of the control Q4 genomic DNA were readily digested with *Nco1* ; data not shown). Curiously, the Q4 allele was not amplified from any of the extracts despite the fact that F1 progeny were backcrossed to Q4 in the crossing regime.

Two out of three clones derived from the first extract, and two out of two clones derived from a second, contained polymorphisms characteristic of the Q4 allele at positions 303 and 345 and the polymorphism characteristic of RM-8 at position 410. On the other hand, the third clone derived from the first extract and a single clone derived from the third extract contained all three polymorphisms characteristic of RM-8. Again, no fragments generated entirely from the Q4 chromosome were found among the cloned DNAs.

Conclusions:

1. The Q4 allele was not amplified from any of the extracts despite the fact that F1 progeny were backcrossed to Q4 and would therefore be expected to contain at least one copy of the Q4 fourth chromosome.

2. Progeny homozygous for the Q4 allele would be expected if the malathion resistance mutation was located on a gene separate from *LcαE7*. No such progeny were found.

3. No MCE low/E3 staining (presumably susceptible to both malathion and diazinon) progeny were recovered, which would be expected if E3 and MCE were separate genes.

4. At least two of the extracts contained a fourth chromosome that was a Q4/RM-8 recombinant somewhere in the region of the *LcαE7* gene 5' to the Gly to Asp mutation at nucleotide position 410. The origin of flies carrying this fourth chromosome and the MCE activity / PAGE phenotype of the resultant chimeric protein are unknown.

5. It is clear that none of the putative E3/MCE recombinants were the outcome of simple reciprocal recombination events during the crossing programs; they do not, therefore, constitute proof that the E3 and MCE genes are separate genes.

It is clear from the present invention that malathion resistance in *L. cuprina* is the result of a structural mutation in the LcαE7 (E3) gene, the same gene which mutates to give resistance to diazinon/parathion type OPs. In other words, the MCE and E3 genes are probably allelic and not separated by 0.7 map units as previous classical genetic studies had indicated. The allelism of the two resistance mutations explains the observation of Smyth, K-A.,Russell,R.J. and Oakeshott,J.G. (Biochemical Genetics 32:437, 1994) that there is a negative association between malathion-type OP resistance and diazinon/parathion type OP resistance. The presence of malathion-type resistance alleles in a population would therefore suggest the use of diethyl

OPs for combating flystrike, while the presence of diazinon/parathion-type resistance alleles would suggest the use of dimethyl OPs.

The MCE enzyme produced by the method of the present invention may be used to develop a functional *in vitro* assay for the degradation of carboxylester and dimethyl type OPs in much the same way as the E3 enzyme (Patent: Enzyme Based Bioremediation) has been used to develop an *in vitro* assay for the general OP hydrolysis. Used together, such assays provide scope for screening for alternative OPs that might overcome resistance problems by E3-like enzymes.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.